RESULTS

A lymphocyte proliferative response was observed in cultures incorporating supernatants from cultured cells derived from perilesional tissues (median stimulation index 17.5, range 9.8–36), in contrast to normal controls (median stimulation index 1.3, range 0.97–1.8), p = 0.012, Mann-Whitney U test. Similar responses were observed with supernatants derived from both dermal papilla cells and interfollicular fibroblasts. This occurred irrespective of the source of responder cells and whether autologous or allogeneic combinations of dermal papilla cells and responder cells were used. No significant proliferative effect occurred in experiments using media from cultures derived from non-lesional sites in alopecia areata patients, or from normal control subjects.

Significantly higher levels of IL-6 were detected in supernatants derived from perilesional dermal papilla cells (median level 55 ng/ml, range 6.7–440) and in one cell line from a non-lesional site than in those from normal dermal papilla cells (median level 3.0 ng/ml, range 2.6–8.5), p = 0.037, Mann-Whitney U test. Biologic activity of the IL-6 in these supernatants was confirmed by neutralization with polyclonal goat anti-human IL-6 antiserum prior to performing the B9 assay. When the lymphocyte proliferation assay was repeated using supernatant samples pre-incubated for 1 h with neutralizing IL-6 antiserum, there was only a small reduction in the proliferative response that was not significant (analysis of variance), suggesting IL-6 was of only modest importance in inducing the mitogenic effect observed. In normal hair follicles, staining with the Mx antibodies was confined to the inner root sheath. Increased staining for Mx protein was observed within the hair follicles of perilesional alopecia areata tissues only. The distribution of increased Mx expression was mainly in the keratinocytes of the outer root sheath and matrix of lesional anagen follicles but was mildly increased also in the dermal papilla compared to normal scalp tissue.

DISCUSSION

These experiments showed that cultured dermal papilla cells and interfollicular dermal fibroblasts from perilesional sites in alopecia areata release soluble factors that stimulate lymphocyte proliferation. Biologically significant levels of IL-6 were identified in dermal papilla cell culture supernatants derived from cells up to passage 9 showing this property can be maintained for long periods *in vitro*. The observation of Mx protein expression signifies local production of type I interferons in the hair follicle in alopecia areata. The antiproliferative activity of these cytokines could well be relevant to the known pathodynamic disturbances of the hair-growth cycle including precipitation of catagen in newly involved follicles and failure to advance beyond the anagen III–IV stage in bald areas.

The likely role of cytokines in the pathogenesis of alopecia areata can be summarized in two hypotheses. First, cytokine gene dysregulation is a possible mechanism for the primary disease abnormality in alopecia areata. Increased cytokine production by the dermal papilla of the hair follicle could explain the observed inhibition of hair growth, disturbance of the hair-growth cycle, and the inflammatory response. In a patient with the appropriate genotype, the disease process could be triggered by diverse stimuli including infection, trauma, and stress, all of which have been implicated clinically. Alternatively, production of pro-inflammatory cytokines and chemoattractant factors by the dermal papilla may be central to the pathogenesis of alopecia areata, although not itself explaining the basic cause of the disease. The increased cytokine production found in alopecia areata may be a manifestation of a final common pathway of disturbances found in a variety of diseases.

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Autoimmune Diseases: Promising Emerging Therapies

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Alopecia Areata (AA), a condition involving patchy to complete hair loss, is marked by mononuclear cell infiltration in and around the hair follicles, as well as HLA associations, cytokine patterns, and abnormalities in follicular cells. These features, coupled with some clinical improvement after treatment with anti-inflammatory agents, suggest an autoimmune component in AA. This presentation will describe pathogenic mechanisms and therapeutic strategies under development in other probable autoimmune diseases that may be instructive in the understanding and possible treatment of AA. Two excellent review articles on this subject have been published recently by Steinman [1] and Miller and Karpus [2].

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PATHOGENIC MECHANISMS

In neurologic diseases such as experimental autoimmune encephalomyelitis (EAE) and multiple sclerosis (MS), the pathogenic agent

is known (EAE) or thought (MS) to be an activated Th1-type cell specific for myelin antigen epitopes. Similarly, T cells directed against organ-specific antigens are thought to initiate inflammation in other diseases such as arthritis and diabetes. It is widely believed that the autoimmune process involves an initial exposure to some exogenous agent (e.g., a latent, tissue-specific virus) that later, upon activation by stress or other causes, induces local damage that sensitizes virus-specific T cells, autoreactive T cells, or cross-reactive specificities. These T cells, upon exceeding a critical threshold frequency, cross the blood-tissue barrier and initiate the inflammatory cascade after reactivation with viral or tissue antigens. Reactivation induces the release of pro-inflammatory cytokines such as interferon-γ and tumor necrosis factor (TNF) that respectively upregulate major histocompatibility complex (MHC) expression (and inflammatory potential) and target cell damage. Additional mononuclear cells, including monocytes and tissue macrophages, are recruited to the area and activated by lymphokines. The key elements to the development of chronic progressive disease appear to be the presence of persistent antigen in association with MHC class II molecules, and maintenance of a critical frequency of inflammatory T cells. The distinction between disease induced by T cells specific for autoantigens versus persistant viral antigens is not always clear. In the case of Theiler's murine encephalitis virus, clinical impairment and progressive demyelination are mediated by virus-specific T cells without detectable involvement of autoantigens. However, in organ-specific disease models, disease progression may be maintained entirely by autoreactive T cells. The identification of relevant target antigens is complicated, however, by "determinant spreading," that is, sensitization to new pathogenic epitopes unmasked during the attack on the initiating antigens [3]. In AA, it is not as yet possible to clarify the respective roles of viruses versus autoantigens in the pathogenic mechanism.

THERAPEUTIC STRATEGIES

Understanding of pathogenic mechanisms has led to both nonspecific and antigen-specific therapeutic approaches. A number of exciting new approaches that may have application to the treatment of AA are discussed below.

Anti–TNF-α Antibody Because of the key role of TNF-α in arthritis, a chimeric anti–TNF-α monoclonal antibody was injected into patients with rheumatoid arthritis, and was found to produce significant clinical improvement [4]. This approach might have immediate application to AA, because TNF-α has been found in inflamed follicles, and could be partly responsible for the abnormalities in dermal papilla cells.

Agents Directed at the T-Cell Receptor (TCR) The overexpression of Vβ8.2 by TCR specific for basic protein (BP) by H-2^u mice with EAE allowed successful treatment with Vβ8.2-specific monoclonal antibody [5,6]. The putative role of T cells directed at BP as pathogenic agents in MS has led to an application of the T-cell vaccination principle first developed by Irun R. Cohen. The most effective study in humans demonstrated that two to three injections of attenuated, autologous BP-specific T-cell clones could induce cytotoxic anti-idiotypic T cells that deleted BP-specific T cells from the periphery [7]. Our own studies utilized TCR CDR2 peptides to induce anergy in encephalitogenic Vβ8.2+ BP-reactive T cells and reverse EAE [8]. More recently, we showed that Vβ5.2 and Vβ6.1 CDR2 peptides could significantly increase the frequency of regulatory TCR peptide-specific T cells in MS patients, with a corresponding loss of BP reactivity, and possible clinical benefit [9]. Therapies directed against the TCR require knowledge of the target antigen, and are not yet applicable to AA, in which there is still no defined tissue-specific antigen.

Antigen-Driven Tolerance Tolerance induced to specific autoantigens represents the most selective approach for immunoregulation. High-dose tolerance, induced by injecting antigen repetitively, deleted BP-reactive T cells and ameliorated EAE [10]. Soluble MHC/Ag complexes, made by combining encephalitogenic epitopes of BP with soluble MHC class II restriction molecules, provides specific activation of the TCR complex without co-stimulatory interactions. This approach induced clonal anergy and could successfully treat EAE [11]. Recently, this concept was extended elegantly through the production of a soluble, recombinant MHC II molecule linked covalently to a chosen peptide ligand [12]. Similarly, antigen-presenting cells crosslinked chemically with crude central nervous system antigens anergized Th1 cells and reduced the number and severity of relapses in EAE [13]. This technique has the advantage of not requiring knowledge of the specific target antigens within the affected organ, and conceivably could be applied to AA using follicular extracts. A new and highly selective approach involves designing altered peptide ligands that mimic the T-cell determinant, but act as T-cell antagonists [14]. TCR antagonist peptides have been effective in EAE [15], but have no application to AA until disease-associated epitopes can be identified. Orally induced tolerance protocols may induce clonal deletion or active suppression, and have been highly effective for suppressing EAE [16,17] and subsequent trials in MS using orally administered bovine myelin inhibited BP responses with possible clinical benefit [18]. Because the target autoantigen does not need to be known, this approach may be applicable to AA.

CONCLUSIONS

There are four conclusions: growing knowledge of autoimmune disease mechanism allows novel intervention strategies; different strategies have varying degrees of therapeutic potential, and combination therapies may be useful; successful intervention will be necessary to establish the contribution of autoimmunity to pathogenesis; and early intervention may prevent epitope spreading.

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Abnormalities in the Ultrastructure of Melanocytes and the Outer Root Sheath of Clinically Normal Hair Follicles from Alopecia Areata Scalps

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A subclinical condition of alopecia areata has been described [1], in which abnormalities seen in follicles from active alopecia areata were also found to some extent in follicles from clinically normal, that is non-balding, regions of alopecia areata scalps. Ultrastructural examination of the hair follicles from non-balding regions of alopecia areata may reveal important information about the changes involved and help indicate the etiology of the disease.

Our previous investigations have identified abnormalities in the orientation of the cells of the dermal papilla [1] and evidence of ultrastructural abnormalities‡§ within the cells and of the dermal papilla–epithelial junction in follicles from clinically normal areas of alopecia areata scalps. Melanocytes have been implicated in the etiology of alopecia areata and regrowing hairs are often white [2]; because abnormalities of melanocytes and an unusual outer root sheath distribution have been reported [3] we have extended our investigations to the melanocytes and epithelial components of the clinically normal follicle.

We have compared the ultrastructure of clinically normal hair follicles with those of normal control scalps and active lesional edges to determine the earliest changes at the ultrastructural level that may indicate the primary site of damage in the alopecia areata follicle. Scalp biopsies (4 mm) were taken from five normal controls and from six alopecia areata patients on first presentation. Patient biopsies were taken from the "active" edge of a patch of alopecia areata and a non-balding area within the same scalp bearing clinically normal terminal hair. Control and non-balding region biopsies were taken from the occipito-parietal region. Individual follicles were microdissected, processed routinely for electron microscopy, and sectioned longitudinally through the middle plane of the follicle [4].

We have examined the ultrastructure of the hair bulb adjacent to the upper regions of the dermal papilla and found that all follicles contained melanocytes in the undifferentiated matrix and presumptive cortical regions. Normal follicles always had well developed melanocytes with melanosomes actively engaged in pigment transfer. In follicles from active alopecia areata regions melanosomes were generally absent or were small and poorly differentiated. Follicles from non-balding regions either contained well-formed melanocytes that were actively engaged in pigment transfer when there were few or no other signs of cellular degeneration or, conversely, showed varying degrees of abnormality of melanocyte distribution and morphology.

We have not seen the unusual presence of melanocytes in the outer root sheaths as described by Tobin *et al* [3] in any follicles from alopecia areata, but we have noted other degenerative tendencies that correspond well with the hypothesis of a sub-clinical condition in the disease. The cells of the outer root sheath were vacuolated to some extent in all follicles, outermost cells more so than inner. This vacuolation is attributed to loss of glycogen during preparation for microscopy [5]. Non-balding region follicles resembled normal follicles below about half papilla height. However, above half papilla height there was evidence of more extensive vacuolation and most noticeable was the marked presence of a fine granular deposit. In follicles from active lesions of alopecia areata vacuolation was even more pronounced, but no granular deposits were observed.

Overall, degenerative trends in the structure, composition, and activity of melanocytes and melanosomes appeared to be dependent on the degree of activity of the disease. In the outer root sheath of non-balding region follicles cells were deteriorated and granular deposits were seen, whereas the inner epithelial cells appeared to be in quite good condition. The fine granular deposit observed in the follicles is probably glycogen. Small amounts were detected in some normal hair follicles, where it was located mainly on the inner edge of the innermost cells of the outer root sheath. However, in follicles from non-balding regions much larger amounts of granular deposit were distributed over a much greater area of the outer root sheath. A possible explanation for this increased content and

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